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The photo-stimulatory effect of low level laser therapy on the proliferation rate of human monocytic leukaemia cells

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Abstract

Low-level laser therapy (LLLT) is a form of phototherapy used to promote cell proliferation. This study investigates the potential role of LLLT in cellular proliferation of human monocytic leukaemia cells (THP-1) under in vitro conditions. Cells were irradiated with an 850 nm diode laser and exposed to doses ranging from 0 – 26.8 J/cm². After irradiation, cells were incubated for 12 hr and 24 hr to allow time for proliferation. Comet assay was conducted to evaluate genotoxicity of the irradiated cells. Trypan blue was used to estimate cytotoxicity, which peaked at the highest dose as expected. Preliminary results suggest that cell counts increase at low doses, whereas a decrease in cell number at high doses was noted compared to controls. Comet assay showed no significant difference between irradiated and non-irradiated cells at low doses. In contrast, DNA damage increased at doses ≥ 8.9 J/cm² and was comparable to the 100 μ M H₂O₂ positive control at the highest fluence. It could be concluded that LLLT has the ability to stimulate the THP-1 cell line to proliferate if supplied with the correct energy and dose.

1. Introduction

Low-level laser (light) therapy (LLLT) is an important technique to treat a plenty of circumstances that require energizing of healing, relief of pain and inflammation, and restoration of function [1]. Master was the first who introduced (LLLT) for potential clinical applications in the late 1960s [2]. Low intensity, non-thermal irradiance does not generate heat [2]. This process is also referred to as photostimulation, phototherapy or photobiomodulation [3, 4].

The biomodulatory effects of LLLT have been disseminated in variety of in vitro studies using different cell types such as fibroblasts, lymphocytes, keratinocytes, macrophage, HeLa and Stem cells [4]. It has been demonstrated that LLLT has the ability to modulate the process of tissue repair by prompting of cellular reaction such as migration, proliferation, apoptosis and cellular differentiation [5].

Low power lasers have been shown to be safe, non-invasive and highly beneficial in various fields of medicine, including dentistry and orthopedics (Cobb, 2006) [3]. However, the mechanism of photobiostimulation by LLLT is poorly understood. It seems that LLLT can be influential at the molecular, cellular and tissue levels [6]. The basic biological action of lasers is the absorption of red and NIR light by specific photoreceptors, such as cytochrome c oxidase (CCO). This chromophore is the fourth protein of five proteins located in the respiratory chain within the mitochondria, and possibly also photoreceptors in the plasma membrane of cells; so, a series of events occur in the mitochondria, producing biological alterations for various processes [7]. Studies have been conducted to record the absorption spectra of CCO in different oxidation states, which is found to be identical to biological responses to LLLT. Light absorption may lead to photodissociation of the restrained nitric oxide from CCO, leading to the boost of enzyme activity, increasing electron transport, oxygen consumption, mitochondrial respiration, and ATP production. Thus, altering the mitochondria or redox state of cell, LLLT can stimulate the activation of many intracellular

signalling pathways and changes of cellular activities; including proliferation, regeneration and cell survival [4, 8] .

Many different types of laser light sources, including diode lasers, helium-neon (He-Ne), ruby, and gallium-aluminium-arsenide (GaAlAs) lasers are used to deliver LLLT in different medical applications.

In LLLT, energy is delivered to a biological system at low levels, and therefore does not generate significant heat [7]. Studies regarding the biostimulation effect of LLLT found no increase in heat of the target tissue, and this can be ignored if the induced temperature rise is $< 1^{\circ}\text{C}$ [9]. Many investigators have shown that in fibroblast suspensions there is no temperature change with LLLT irradiation [10, 11] . Other researcher, Schneede et al. [12], is in agreement with these findings, he found the increase in temperature to be $< 0.065^{\circ}\text{C}$, during laser irradiation of $40\text{ mW}/\text{cm}^2$, using a microthermal probe in a monolayer of cells[12] . In contrast, the surgical lasers of high energy (e.g., carbon dioxide lasers, CO_2 and neodymium-YAG lasers, Nd: YAG) are able to elevate the tissue temperature high enough to cut , cauterise, vaporise and sterilise it.[13].

2. Experimental methods

2.1 Experimental techniques for the design and fabrication of the Biological Effect of Laser Therapy (BELT).

In order to study the Biological Effect of Laser Therapy (BELT) at the cellular and molecular level, an instrumental platform for BELT was designed and built in Plymouth during the course of this work. Figure 1 shows the schematic designed. The 850 nm laser source has a polarised circular beam, with variable output power (up to 25 mW). The beam expander increases the beam diameter to match the size of the well containing the cells. The polarising beam splitter enables 'p' or 's' polarised light to be delivered to the cells, according to the

orientation of the laser diode. In this work 'p' polarised light was used. The 'p' polarised light passes through a series of beam splitters, delivering 40%, 30%, 20% and 10% of the input beam to four wells which contain the cells in suspension. In the work reported here, only 40% of the laser power was delivered to the cells. The laser power was fixed and the exposure time varied to expose the cells to the different doses. The 30%, 20% and 10% options are part of the system design and will be used for future experimental studies, which will also include 's' polarised light.

The laser diode output was calibrated using a power meter (at 850 nm) in parallel with the in-built power monitoring capability of the laser diode. Subsequently power delivered was determined from in-built power monitor diode. A stopwatch controls the exposure time, which is the only variable. The uncertainty in exposure time was ± 1 s.

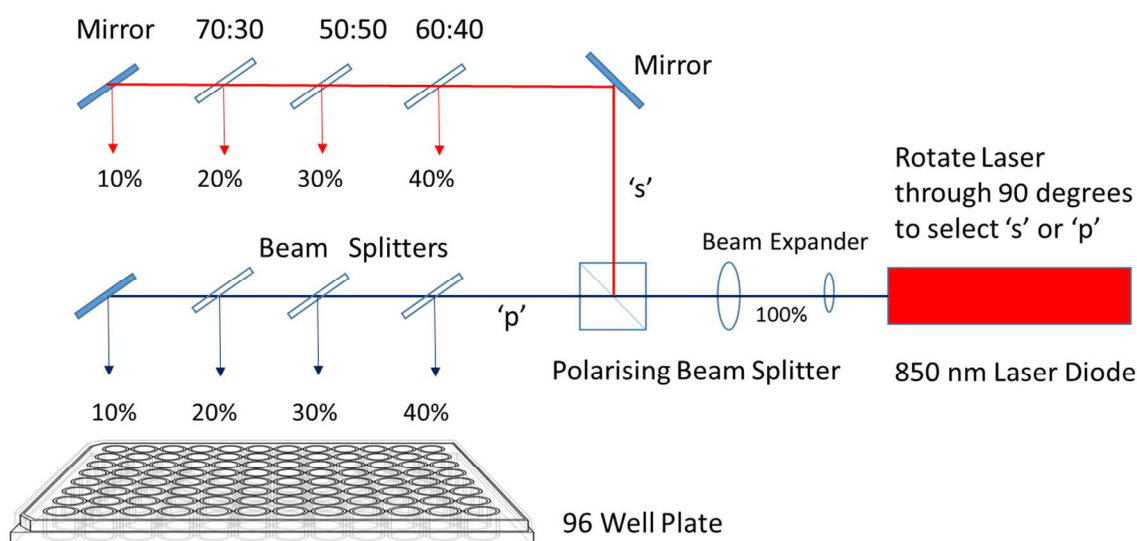


Figure 1 Schematic experimental layout

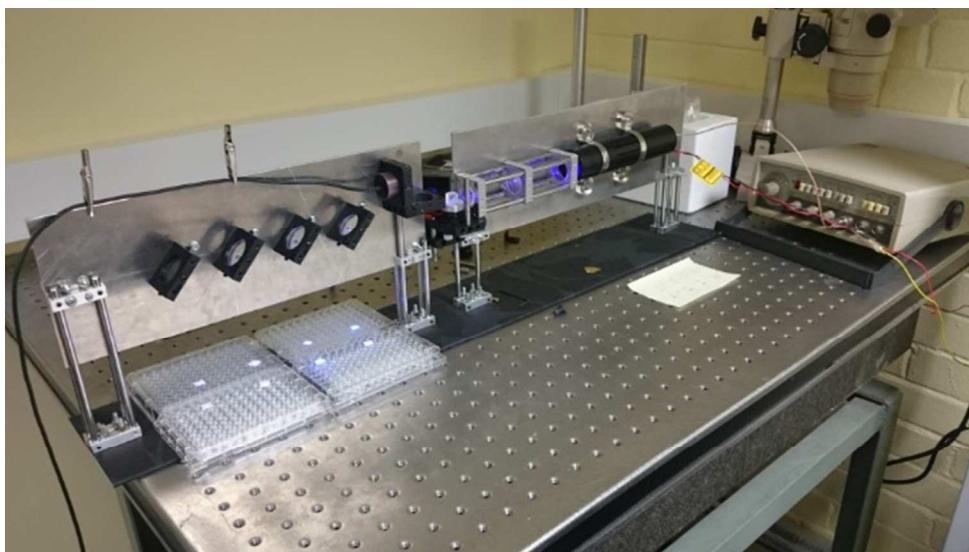


Figure 2 The experimental BELT system.

2.2 Cell cultures

The human monocytic leukaemia THP-1 cell line, permanent cell line, derived from the peripheral blood of a one year old male with acute monocytic leukaemia. THP-1 were maintained, as described by Chia.et al, (2014) [14], in RPML-1640 (Lonza Bioscience Ltd, UK) complemented with 10% foetal calf serum FBS (Labtech, UK), 5ml of L-glutamine (Lonza Bioscience Ltd, UK) and 5ml penicillin/streptomycin(Source Bioscience 1, UK). THP-1cells were grown in 50 ml culture flask, the flask containing 20ml of medium plus cell, at 37°C with 5% CO₂ in a humidified incubator. The cells were sub-cultured approximately every 4 days by changing the media at the ratio of 1:4, and the cell density was counted after every subculture by the light microscope.

2.3 Laser irradiation

The laser device used in this study was the diode laser model APMT25 (850-40)/5342 (Power Technology Incorporated, Alexander, AR USA) with a wavelength of 850 nm and power output of up to 27 mW. The light spot was delivered with diameter of 8.7mm in the continues wave (cw) mode. The laser

spot with respect to the cell well is shown in Figure 3a. It was assumed that the laser intensity was constant across the spot diameter. The laser power delivered was constant at 11.1 mW, giving a power density of 34.6 mW/cm². After accounting for the fact that 86 % of the power reaches the cells in the well, the laser power becomes 9.5 mW with a power density of 29.6 mW/cm². The laser intensity, and hence temperature, is not constant across the cells, and can be considered to be represented by the 'bands' shown in Figure 3b, with T₀ representing the maximum temperature. In this work the mean delivered power was used, with the only variable being irradiation time, as in Equation 1 below. The determination of the temporal and spatial distribution within the well is currently being investigated and the results will published soon.

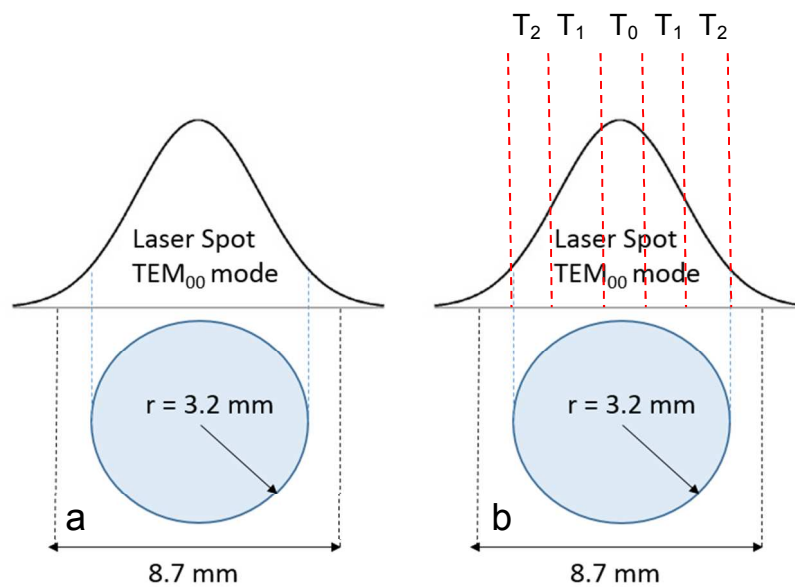


Figure 3a The diode laser beam is expanded to deliver stimulating light to the full well area. **Figure 3b** Temperature bands arising from the Gaussian intensity distribution.

The dose was calculated as:

$$\text{Dose} = \text{Power Density} \cdot \text{Irradiation Time} = \frac{\text{J/s}}{\text{cm}^2} \cdot \text{s} = \text{Jcm}^{-2} \dots (1)$$

THP-1 cell line were transferred to 96 well plate with cell density of (10,000 cells / 200 µl) to be irradiated by the diode laser for the durations of 20, 40, 120, 300, 600 and 900 sec for different fluences of 0.6, 1.2, 3.6, 8.9, 17.8 and 26.8 J/cm², respectively with one group of cells non-irradiated as control. Determination of cell proliferation, cell viability and comet assay were achieved immediately after irradiation and at 12 hr and 24 hr incubation post irradiation, Figure 4.

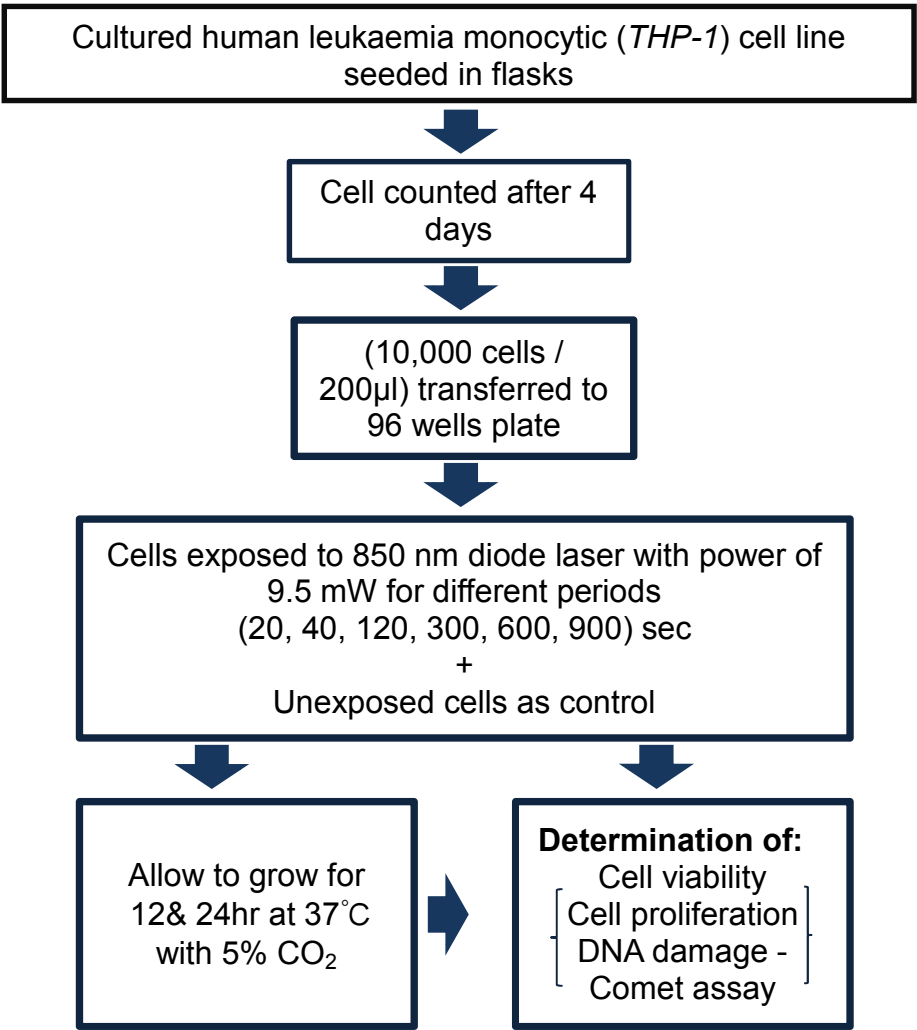


Figure 4. *In vitro* studies experimental overview

2.4 Proliferation rate and cell viability

The proliferation rate and cell viability of the cultured THP-1 cell line was determined by using Trypan blue stain 0, 12 and 24 h after laser treatment. This method depends on membrane integrity to recognize the viable and non-viable cells: only non-viable cells are stained by the blue dye. For this process 20 μ l of cell suspension is mixed with 20 μ l of 0.4 M Trypan blue solution, incubated for 10 minutes at room temperature to be counted by the light microscope.

2.5 Comet assay for DNA damage

Comet assay as commonly known or single cell gel electrophoresis (SCGE) is a technique permits the detection of DNA damage in single cells. Comet assay was performed as described in Raisuddin and Jha. (2004) with some modifications. Microscope slides were pre-coated with normal melting point agarose (NMPA; 1.5% in TAE (0.04M Tris-Acetate, 0.001 M EDTA, pH 8)) and kept in 37°C incubator before used. 10 μ l (10,000 cells) of cell suspension was transferred into eppendorf and suspended in 75 μ l of low melting point agarose (LMPA; 0.75% in PBS) and two 75 μ l drops were dispensed onto each marked slide and cover slipped (to produce two replicate microgels), slides were left in cold room at 4°C for 1h to allow gels to set. Removing the coverslips gently and arranging the slides back to back into coplin jar containing lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% N-lauryl-sarcosine, 1% Triton X-100, 10% DMSO, pH adjusted to 10 with NaOH) and incubated for more than 1 h at 4°C. Following lysis, for allowing time for DNA to unwind, slides were transported to an electrophoresis chamber (switch off), filled with 545 ml of chilled electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH 13) at 4 °C for 20 min. Then the chamber switched on (21 V/ 620 mA) to allow electrophoresis to run for 25 min. After electrophoresis, slides were transported to a new jar containing neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for 2 min, twice, and finally rinsed twice with distilled water and left to dry before scoring.

To ensure unbiased scoring, cells on the slides were scored randomly, using a comet image analysis software program. 20 μ l of 20 μ g/ml ethidium bromide was added onto each replicate microgel to be stained, and 50 cells per microgel (100 per slide) were scored. Scoring of slides were performed using an epifluorescence microscope using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) and imaging system (Comet IV, Perceptive Imaging, UK). Comet assay software packages record a number of different parameters, with % tail DNA considered the most reliable [Kumaravel TS and Jha AN 2006] [15]. Hence, comet assay results are reported as mean % tail DNA.

2.6 Statistical analysis

All data acquired were tested for normality using Anderson-Darling test and Shapiro-Wilk and for equal variance with Brown-Forsythe test, and appropriate parametric or non-parametric tests were used. Where appropriate, regression and correlations were determined using Pearson's correlation coefficient. Analysis was made using a 2-way ANOVA with Holm-Sidak for pairwise comparisons and comparisons versus a control group, and Tukey's pairwise comparisons as post hoc tests, and for nonparametric Kruskal-Wallis test for pairwise comparisons and Mann-Whitney for comparisons versus a control group.

For the comet assay data, the median was calculated for % tail DNA values for each slide. The resulting data set based on medians was non-normally distributed and was analysed by Kruskal-Wallis test for pairwise comparisons and Mann-Whitney for comparisons versus a control group. Statistics were calculated using SigmaPlot Statistics (version 13.0.0) and Minitab (version 17.1.0; Minitab Inc., State College, PA, USA). Significance for all tests was set at $p \leq 0.05$.

3. Results

3.1 Effect of LLLT on cell proliferation

Proliferation of THP-1 cell line has been determined by using microscope and haemocytometer, Figure 4. Cells irradiated with 850 nm diode laser at fluences of (0.6, 1.2, 3.6, 8.9, 17.8 and 26.8 J/cm²) showed a remarkable change in cell count, and hence proliferation, as compared to the non-irradiated cells. Comparing the effect of different fluences showed a noteworthy increase in proliferation at low doses (0.6, 1.2 and 3.6 J/cm²), and decreased proliferation at higher doses (8.9, 17.8 and 26.8 J/cm²) after 12 and 24 hr post irradiation as compared to control (the non-irradiated cells). A 2-way ANOVA with Tukey's pairwise comparisons as post hoc tests showed a significant change in cell viability for different laser doses $P < 0.05$.

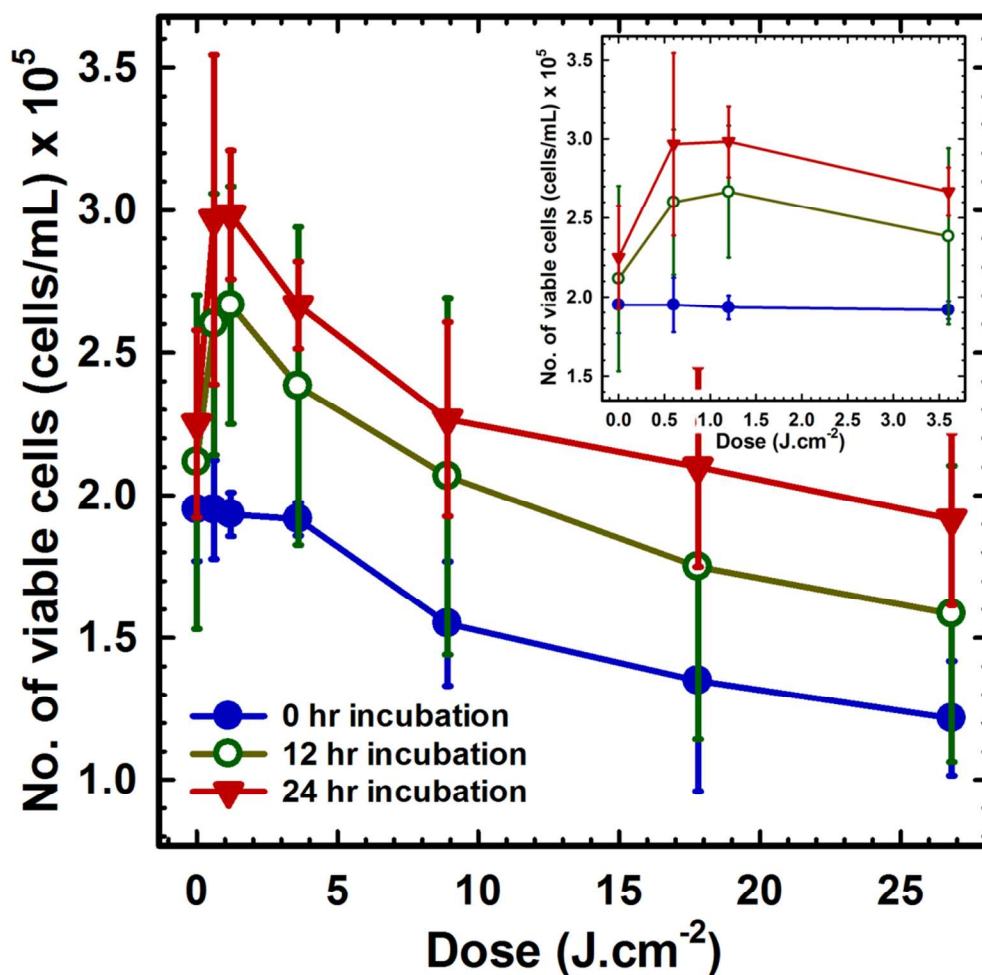


Figure 5 Number of viable THP-1 cells after irradiation with diode laser. (•) Immediately post irradiation. (◦) After 12 hr incubation post irradiation. (▼) After 24 hr incubation post irradiation. Each point represents the mean of three separately performed assay. *Error bars*, SEM. A significant difference in viable THP-1 cells number between the irradiated and non-irradiated cells are indicated ($P < 0.05$). The inset shows in detail the data very low doses.

3.2 Effect of LLLT on cell viability

Cell viability was assessed in order to determine cytotoxic events taking place in the cell immediately after irradiation, twelve hours or twenty four hours incubation after irradiation. A 2-way ANOVA with Holm-Sidak test for pairwise comparisons and comparisons versus a control group showed a significant change in cell viability for different laser doses $P < 0.05$.

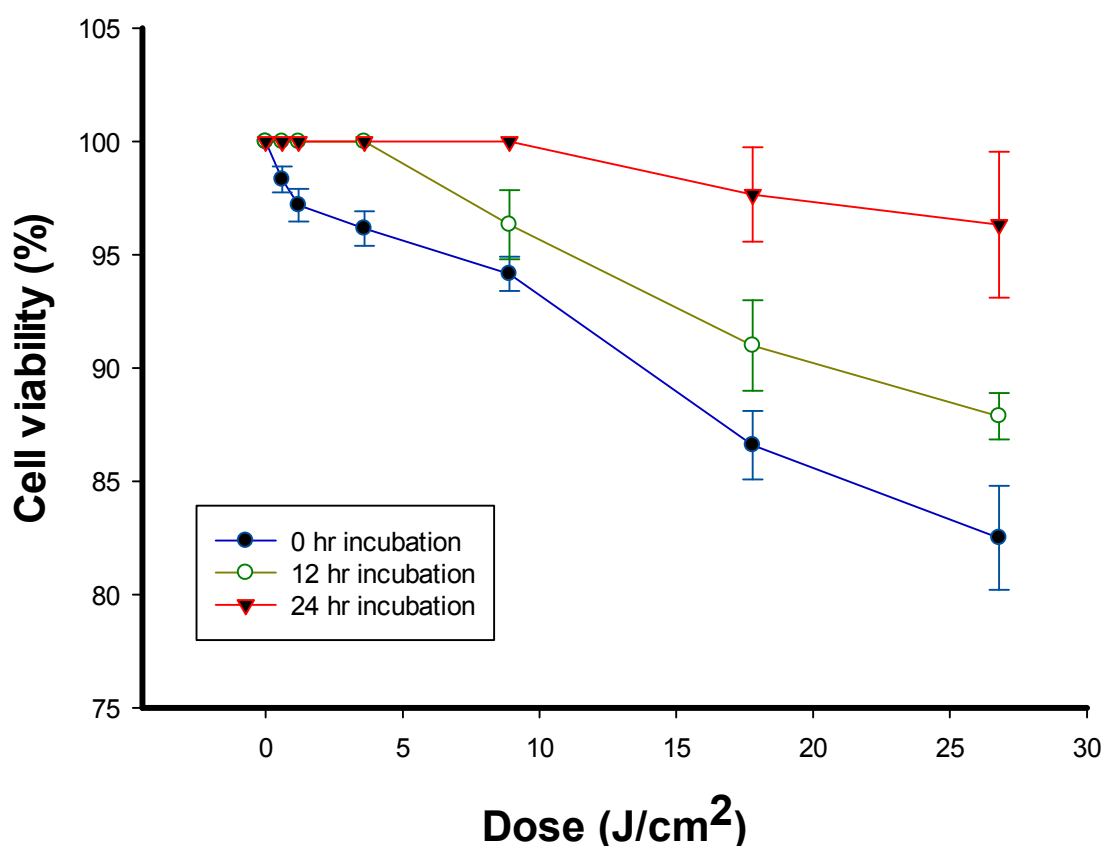


Figure 6 Viability of THP-1 cells after irradiation with diode laser. (•) Immediately post irradiation. (◦) After 12 hr incubation post irradiation. (▼) After 24 hr incubation post irradiation. Each point represents the mean of three separately performed assays. Error bars, SEM. A significant difference in viable THP-1 cells number between the irradiated and non-irradiated cells are indicated ($P < 0.05$).

3.3 Effect of LLLT on DNA

Representative images of THP-1 cells after SCGE are shown in Figure 7. Through the electrophoresis step cells with elevated levels of DNA damage display the distinctive 'comets' due to negatively charged DNA fragments moving away from the nucleoid towards the anode.

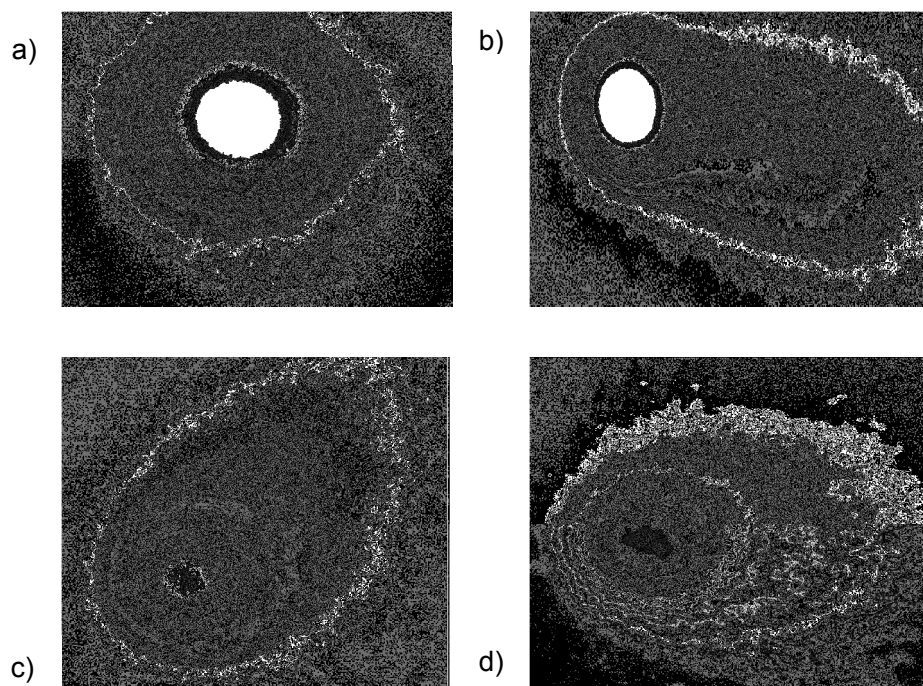


Figure 7. THP-1 cell lines showing increasing damage of DNA as detected by the SCGE (Comet assay) after exposure to different doses of LLLT; (a) Unirradiated cells (b) irradiated cells for low doses (c) and (d) irradiated cells for higher doses.

The effect of LLLT on DNA damage of THP-1 was dose-dependent. A significant increase in percentage tail DNA for all doses (Fig. 8; Kruskal-Wallis test, $P < 0.05$). A significant dose-dependent increase was observed between the high doses (4-26.8) J/cm^2 and the control and the low doses (0.6-4) J/cm^2 , while insignificant

increase between the low doses and control (Fig. 8; Mann-Whitney U tests, $P < 0.05$), Figure 7.

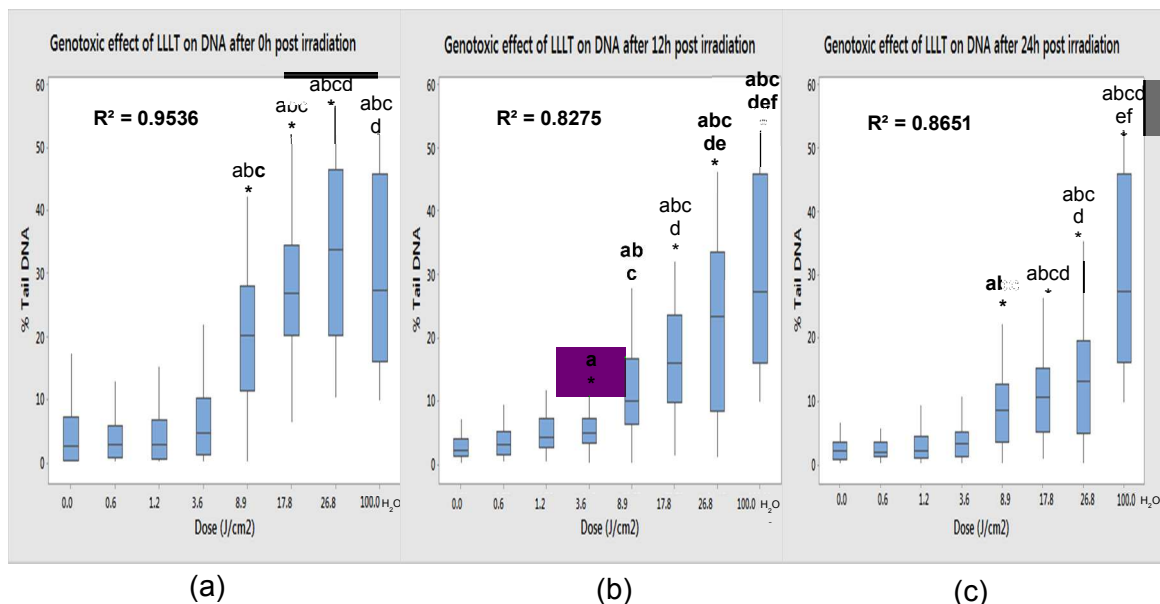


Figure 8. The genotoxic effects in THP-1 cells after *in vitro* exposure to LLLT, unirradiated (control) samples used as negative control and 100µm H₂O₂ as positive control. (a) Immediately post irradiation, (b) 12 hr incubation after irradiation, (c) 24 hr incubation after irradiation. Asterisks refer a significant difference from the control ($P < 0.05$); mismatched lower case letters refer significant differences between treatment groups ($P < 0.05$). Kruskal Wallis tests for the three time points gave p values of 0.008 (immediately post irradiation), 0.007 (12 hr) and 0.013 (24 h). Pearson's correlation statistics are shown on each graph.

4. Discussion

Cell proliferation is a significant technique widely utilised to explore the impacts of LLLT irradiation [16]. Cell proliferation, adhesion, as well as DNA and RNA synthesis are the most cellular responses studied most consistently [17]. The result in the present study clearly confirm for the first time that low level laser irradiation significantly promotes the proliferation rate of THP-1 cell line. The extant of boost of the cell proliferation of THP-1 cells for 0, 12 and 24 hr

incubation post the laser exposure was higher at low doses than high doses. Whereas, an inhibitory effect was indicated at high doses irradiation. The results of the present study support those of previous studies, which emphasised the role of therapeutic lasers with low doses in enhancement of proliferation of different human cell types in vitro through 24hr post-laser-application. Frozanfar et al., (2013) [18] reported on the effect of low energy laser with $4\text{J}/\text{cm}^2$ at near-infrared region (NIR) on the proliferation of human gingival fibroblast cells [18]. Irradiated cells showed slight increasing after day and a significant increase in proliferation rate in two and three day's incubation post-irradiation [18]. Tuby et al. used NIR laser diode to study the photostimulation effect on stem cell proliferation using $1\text{J}/\text{cm}^2$ and $3\text{J}/\text{cm}^2$. The results showed a considerable increase in the rate of cell proliferation in both fluences in compared to untreated control cells, and it found that the irradiation of $1\text{J}/\text{cm}^2$ causes more cells to proliferate than irradiation of $3\text{J}/\text{cm}^2$. No negative impacts of LLLT with these doses on cells in culture have been shown, and, therefore, it suggested that NIR lasers at the power and energy densities used in this study can be safely employed for irradiation of cells in vitro [19]. In a study by Sroka et al. has been shown that the stimulation of proliferation has been related to the irradiation dose. A stimulated proliferation for muscle cell was observed after irradiation with diode laser at a wavelength of NIR and energy density of $4\text{J}/\text{cm}^2$, whereas an inhibitory effect was found after irradiation with $20\text{J}/\text{cm}^2$. In the same study, three different wavelengths at visible and NIR with energy densities in the range of 2 to $8\text{J}/\text{cm}^2$, to find the biostimulatory effect of laser on human breast carcinoma (MCF7) and human glioblastoma (U373) cell lines. The findings revealed that cell proliferation promoted only by low doses [20]. The stimulatory effect of low energy doses has also been studied in fibroblast cultures, using Ga-As laser at IR wavelength and energy density of 3, 4 and $5\text{J}/\text{cm}^2$ to induce proliferation. This study showed a significant increase in fibroblast cell proliferation at energy density of 3 and $4\text{J}/\text{cm}^2$, while that of $5\text{J}/\text{cm}^2$ failed to promote proliferation [21]. Basso et al. used InGaAs (Indium Gallium Arsenide Phosphide) laser irradiation at NIR with energy densities (0.5, 1.5, 3, 5 and $7\text{J}/\text{cm}^2$ to investigate the photo-stimulation

effect in human gingival cells. Twenty-four hours after the laser irradiation showed that (0.5, 3 J/cm²) were the most effective doses to cause observable increase in cell proliferation compared with the nonirradiated group. At doses 5 and 7J/cm², the proliferation of cells was decreased (In Vitro Wound improved by(InGaAsP) [22]. A study for Abrahamse [23], also emphasis that the inhibitory effect of doses ≥ 5 for NIR laser on human cell proliferation. A significant decrease in the number of human adipose derived stem cells was observed after exposed to NIR diode laser at fluencies (5, 10 and 15 J/cm²) [23]. A recent research for Huertas et al. [24] has been illustrated the role of low level laser irradiation fluences on cell proliferation. A positive increase in cell proliferation in human osteoblast (MG63) cells cultured for 24 hr after irradiation by diode laser at intensities of 0.5, 1, and 1.5 W/cm² with fluences of 1–5 J/cm² relative to the control culture, which was not irradiated. At all intensities, the percent of cell proliferation increased and reached a maximum at 3 J/cm², and reduced at high doses [3]. Generally, previous studies pointed out that laser irradiation at fluences up to 4 J/cm² had stimulatory effects, whilst higher energy fluences had negative impacts [24].

On the other hand, studies for other investigators showed results unlike to the previous. Kreisler et al. used NIR laser in the continues wave (cw) mode at energy fluences of 1.96–7.84 J/cm² to evaluate the effect of laser fluences on the proliferation of human periodontal ligament fibroblasts (PDLF) cells. A considerable increase in proliferation activity of cells exposed to the doses up to 7.84 J/cm² compared to the non-irradiated cells [25].

Koutna et al [26], treated a human epithelioid HeLa cell line by NIR diode laser with energy densities ranged from 2 to 99 J/cm² and output power from 72 to 360 mW. The result showed a slight increase in cell number at 1 and 2 days after irradiation, but a clear increasing was observed at 3 day post exposure with all energy fluences [26].

In contrast, Bouvet-Gerbettaz et al [27], did not observe any stimulatory effect of NIR (GaAlAs) diode laser at fluence 4 J/cm² in proliferation of bone marrow cells, they found similar results in control and irradiated cells. Also, Abergel et al [28] indicated the same observation in proliferation of cultured fibroblast after irradiated with both He-Ne and Ga-Al-As lasers [28].

The photon energy is absorbed by intracellular chromophores and converted to metabolic energy, since cellular ATP levels increase almost twofold after irradiation by red to near infrared laser light [29]. ATP works by multiple nucleotide receptor subtypes to increase intracellular calcium concentration (Ca²⁺) and cell proliferation [30-32]. It has been demonstrated that ATP induces activation of the extracellular signal-regulated kinase (ERK) cascade, ERK1/ERK2 in a phosphatidylinositol 3-kinase (PI3K)-independent manner, where both ERK1/ERK2 and PI3K activity (pathways) play an important role in cell proliferation [33, 34].

The damaging effect of low level laser light is controversial. In the present study the cytotoxic and genotoxic effect of the used laser are measured in terms of cell viability and the single cell gel electrophoresis method respectively.

No cytotoxic effect or detectable DNA damage was observed after irradiation with a low fluence laser (< 10 J/cm²), while a slight reduction in cell viability was noticed and a significant increase in % DNA damage post irradiation with higher fluences (> 10 J/cm²) was measured at 0 hr incubation time. However, a significant decrease in % DNA damage at 12 hr and 24 hr compared to 0 hr incubation after irradiation have been shown due to the activation of DNA repair mechanisms. These findings are in agreement with Kujawa [35], who applied near infrared laser light with doses ranging from 3.75 -15.0 J/cm² on B14 cell lines, and found considerable light-induced DNA damage at high doses, while no change in cell survival and no DNA damage was detected at low doses. He concluded that laser therapy with low doses is able to reduce cellular protein damage (protein carbonyl groups) formed by biological oxidant HOCL. Exposure

to laser therapy at high fluences leads to the formation of reactive oxygen and nitrogen species which trigger the initiation of lipid peroxidation, protein damage or DNA modification [36, 37].

Mitochondria is the main source for the production of ATP energy, and the primary producer of the ATP energy is the “electron transport system” of the mitochondria [38]. Electrons that pass through the respiratory chain of the mitochondria, in turn interact with oxygen and hydrogen to produce H₂O and ATP energy in addition to toxic by-products, such as oxygen free radicals are released [39, 40]. Applying low laser therapy with high intensity increases electron transport, and this impedes the flow of electrons through the respiratory chain. This enables an elevated transformation to oxygen, which induces the generation of free radicals. These free radical as known, are highly reactive and can attack the proteins of mitochondrial respiratory chain, DNA and many cell components [38, 40].

Conclusions

LLLT with near-infrared (850 nm) diode laser has the ability to promote the proliferation rate of THP-1 cell line (Fig. 3.1). The results confirm the stimulatory effect of LLLT is dose dependent (Fig. 3.1). As cytotoxicity and genotoxicity occur above a certain dose (Fig. 3.2 and 3.3.2), there is clearly an optimal range of doses for stimulation of tissue and cell lines. Below the threshold of 0.1J/cm² the cells are not stimulated to proliferate, therefore further investigation into optimal conditions for cell stimulation is needed. Identification of the proper treatment conditions for the particular cell lines or tissue is crucial for achieving optimal photobiostimulation. The determination of the temperature rise as well as the temporal and spatial distribution within the well is currently being investigated and the results will published soon. This will add greatly to the understanding of the application of LLLT to the photostimulation of cellular repair processes.

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